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Hantavirus Infection in Taiwan: The Experience of a Geographically Unique Area

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Hantaviruses are rodent-borne viruses, and they, mainly the Hantaan (HTN) serotype, are the causative agents of a group of febrile nephropathies known as "hemorrhagic fever with renal syndrome (HFRS)." Despite the fact that HFRS is frequently reported in China, with an annual incidence of 50,000–100,000 cases, one puzzling observation that no local case of HFRS has been confirmed in Taiwan has yet to be explained. We hypothesized that the hantavirus strain prevailing in Taiwan mainly belongs to the mild strain, the Seoul (SEO) strain, and the absence of severe disease was related to the absence of HTN. To test these hypotheses, this epidemiologic study was performed, including a seroprevalence survey and phylogenetic analysis on hantavirus isolated from the rodent population trapped in major seaports, rural, and mountainous areas of Taiwan. This study also included rodents and viruses from two isolated islands, Kinmen and Matzu, which are geographically adjacent to the east coast of mainland China. There were a total of 5,461 rodents of 16 species captured, and *R. norvegicus* was the most common species, with an antibody prevalence much higher in international seaports (20%) than in rural regions (approximately 5%) and intermediate in some domestic seaports. By reverse transcriptase polymerase chain reaction (RT-PCR), 33.9% of the seropositive *R. norvegicus* were found to have amplifiable hantavirus sequences in their lung tissues, and subsequent phylogenetic analyses indicated that almost all hantavirus in Taiwan was most closely related to the prototype SEO strain, and no HTN strain was recovered from any rodent species indigenous to Taiwan. The seroprevalence of SEO infection in *R. norvegicus* on Kinmen and Matzu was also different from that in southern provinces of China but closely resembled that in seaports in Taiwan, and the SEO identified was genetically linked to Taiwanese SEO strains. These results substantiate our hypotheses, and suggest that the epidemiology of hantavirus infection in Taiwan are different from that in China, where the HTN and SEO strains and HFRS concurrently prevail. ***J. Med. Virol. 60:237–247, 2000.***

INTRODUCTION

The spectrum of infectious disease is changing rapidly in conjunction with dramatic changes in human society and environment [Centers for Disease Control and Prevention, 1994]. Emerging or reemerging infectious diseases have increased worldwide within the past two decades. One of the most deleterious examples was an outbreak of acute respiratory failure with high mortality among previously healthy adults in the Four Corners area of the United States in 1993 [Nichol et al., 1993; Duchin et al., 1994]. The cause of this rapid and lethal incidence was finally confirmed to be a new strain of hantavirus [Nichol et al., 1993], currently re-

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ferred to as Sin Nombre virus (SNV). Since this event, outbreaks of hantaviral disease have been reported in different regions of the world [Childs et al., 1994; Khan et al., 1995; Rowe et al., 1995; Antoniadis et al., 1996; Khan et al., 1996; Schmaljohn and Hjelle, 1997; Bowen et al., 1997; Levis et al., 1998; Papa et al., 1998], and hantavirus infection is now an issue receiving global public health attention.

Hantaviruses are rodent-borne viruses that belong to the family Bunyaviridae. Different from SNV, the common strains of this virus, including the Hantaan (HTN) serotype in Asia, the Seoul (SEO) serotype in cities worldwide or the Puumala (PUU) serotype in Scandinavian and European countries, are the causative agents of a group of febrile nephropathies known collectively as hemorrhagic fever with renal syndrome (HFRS) [Lee, 1996; Nichol et al., 1996]. Depending, in part, on which hantavirus strain is responsible for the illness, HFRS can appear as a mild, moderate, or severe disease. Death rates range from less than 0.1% for HFRS caused by PUU virus to approximately 5% to 10% for HFRS caused by HTN virus. Approximately 150,000–200,000 cases of HFRS involving hospitalization are reported each year throughout the world, with more than half in China [Schmaljohn and Hjelle, 1997; Lee, 1996; Chen et al., 1986; Ruo et al., 1994].

Taiwan was alarmed by the identification of the first imported case of HFRS in April 1995. Subsequent seroepidemiologic investigation unexpectedly revealed prevalent distribution of this virus in rodent populations in Taiwan [Wu et al., 1996; Kao et al., 1996]. Despite this prevailing infection of hantavirus in rodents in Taiwan and despite the fact HFRS is frequently reported in countries around Taiwan, including China, Japan, Korea, and other southeastern Asian countries [Lee, 1996], one puzzling observation has yet to be explained: no local case of HFRS has been confirmed in Taiwan [Wu et al., 1996]. Physicians in Taiwan are generally well-trained and well-informed about hantaviral infection, so the absence of local incidence of HFRS cannot simply be explained by underreporting due to a lack of public health awareness. Instead, it is possible that the hantavirus strain prevailing in Taiwan mainly belongs to the mild SEO strain, and the absence of severe disease was related to the absence of HTN. To test these hypotheses, this epidemiologic study was performed, including a seroprevalence survey and phylogenetic analysis of hantavirus isolated from the rodent population trapped in major seaports, rural, and mountainous areas in Taiwan. Notably, this study also included rodents and viruses from two isolated islands, Kinmen and Matzu, that are geographically adjacent to the east coast of mainland China (5 miles from the China coast) (Fig. 1) but are controlled by the Taiwanese government. The hantavirus strains endemic on these two islands were, therefore, of particular interest, because, since 1949, the only channel for the residents living in these islands to trade or travel has been solely by boat or airplane to and from Taiwan. Geographic location and interrelation among Taiwan, Kinmen, Matzu, and China may provide a unique opportunity for scientific investigation to address the issue regarding the mechanism of hantavirus spread. This study reports such an investigation.

MATERIALS AND METHODS

Rodent Collection

Beginning in 1994, rodents have been systematically trapped at four major international seaports, five domestic seaports, two international airports, two rural areas, and two mountainous regions in Taiwan and on five islands surrounding Taiwan (Fig. 1). The international seaports are located in northern (Keelung), central (Taichung), southern (Kaoshong), and eastern (Hualien) areas of Taiwan, and serve as the major channels of sea transportation and trade between Taiwan and the rest of the world. Rural areas (Chusan and Yuzen) and mountainous regions (Mt. Ali [4000 m high] and Tenlang [3000 m high]) are all remotely located from these international and domestic seaports. The islands of Kinmen and Matzu are located west of Taiwan, and are off the coast of mainland China. However, since 1949, these two islands have been completely isolated from mainland China, and their connection and transportation (including people, animal, and all kinds of goods) have been solely by airplane and boat to and from Taiwan.

Previous reports of HFRS in China and Korea indicate that the endemic seasons for HFRS are usually during the periods of April to July and October to February, and an increase in the hantavirus infection in the rodent reservoir probably occurs one month in advance of these seasons [Lee et al., 1996; Chen et al., 1986; Ruo et al., 1994]. Therefore, we purposely trapped rodents during the period between March and May or between September and December from 1994 to 1995.

Traps were placed in all possible locations where rodents frequently lived. At all sites, 100 traps were set. At each site, traps were set in the afternoon and inspected the next morning for four consecutive days. Rodents in traps were identified as to species and sex and weighed to the nearest gram. They were further anesthetized with ketamine, and their organs (lung, spleen, kidneys, and liver) were aseptically removed. Blood was collected by cardiac puncture. Organs and blood specimens were maintained on dry ice during transportation and were stored at −70°C until assayed at the Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan.

Hantavirus Antibody Testing

Enzyme-linked immunosorbent assay (ELISA) [Childs et al., 1994] was used to detect antibody against hantavirus in rodent sera. Ninety-six well plates were coated with specific hantavirus antigens, which included HTN, SEO, PUU, PH, and SNV, prepared by viral infection with Vero E6 cells. An uninfected Vero-E6 cell culture antigen was also coated to

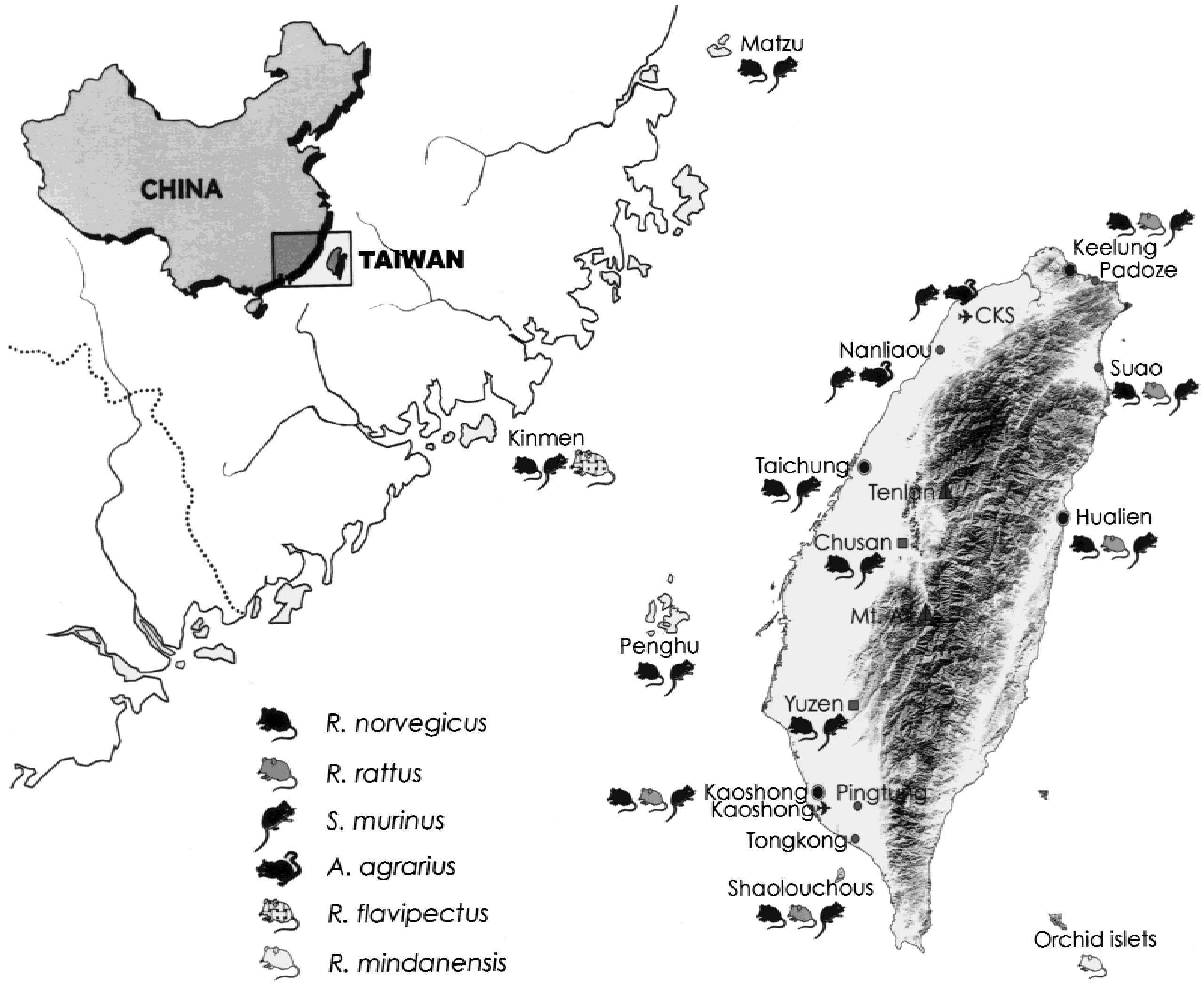

Fig. 1. Map of Taiwan showing the locations where rodents were captured, and the major rodent species captured in these locations.

plates and was used to determine the specific binding of antibodies to viral antigens. Sera from rodents were diluted 1:100 and were added to the coated plates. The specific bound antibody was detected using horseradish peroxidase-conjugated goat anti-rat IgG antibodies and an appropriate substrate (3,3′,5,5′-tetramethylbenzidine dihydrochloride). Optical densities at 410 nm were recorded using a microplate spectrophotometer. The optical density (OD) of the uninfected antigen-coated well was subtracted from that of its corresponding viral antigen-coated well to yield the adjusted OD. An adjusted OD of 0.2 at each dilution was used to assign titers to each serum specimen tested. A titer of 1:400 was considered positive for each antigen. Sera were titrated to a maximum end point of 1:6400. All the ELISA reagents were prepared at the Special Pathogens Branch, Centers for Disease Control and Prevention (CDC), Atlanta, GA and all ELISA's were performed at the Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan.

Rodent RNA extraction, Hantavirus RNA Amplification, and Sequence Analysis

To identify active infection of hantavirus, a RT-PCR assay was used to amplify viral sequences in seropositive rodents. Oligonucleotide primers [Nichol et al., 1993] based upon known hantaviruses were designed for use in the RT-PCR to amplify virus-specific sequences from rodent lung specimens. Total RNA was extracted and purified from rodent lung tissue by an acid phenol procedure, in which approximately 100 mg tissue was homogenized in 500 μl of acidic guanidine isothiocyanate solution. Subsequently, RNA was extracted by phenol/chloroform/isoamyl alcohol. A nested RT-PCR assay was then performed to detect the presence of virus on the basis of two primer pairs specific for two hantavirus serotypes, HTN/SEO and PUU/PH [Nichol et al., 1993]. The primers were located in a region of sequence conservation within the G2 protein-coding region of the M segment of the virus genome [Schmaljohn, 1996]. The 201-bp RT-PCR product was

examined by electrophoresis in a 1.5% agarose gel, and bands of the correct predicted size were excised from the gel and purified. The nucleotide (nt) sequence was determined by an automatic sequencer (ABI370, Perkins-Elmer, Foster City, GA). In this study, all sequences identified were subjected to re-amplification and re-sequencing and further confirmed by sequencing complementary strands, thus eliminating the possibility of creating artifacts by PCR.

Sequences were compared and aligned using the GAP, PILEUP, and LINEUP programs of the GCG software package (Genetic Computer Group, Madison, WI).

Phylogenetic analysis

To define the strain of hantavirus prevalent in Taiwan, and to further identify the possible source of infection, phylogenetic analyses on the basis of the Taiwanese strains and other known strains of hantavirus were performed. Phylogenetic analyses of the nt sequences of different hantavirus strains were done using the maximum parsimony method (PAUP, version 3.1.1) [Swofford, 1991], the maximum likelihood method (PHYLIP, version 3.572) [Shurman et al., 1998], and the distance-based neighbor-joining method (MEGA, version 1.01) [Kumar et al., 1993]. The comparisons of these methods have been briefly mentioned in a previous publication [Levis et al., 1998]. The following previously published hantavirus sequences (accession number) were included in the analysis: HTN 76-118 (Y00386), HTN HV114 (L08753), SEO SR-11 (M34882), SEO R22 (S68035), PUU CG18-20 (M22997), PUU Sotkamo (X61034), PH PHV-1 (X55129), Dobrava (L33685), SNV CC74 (L33474), SNV NMR11 (L37903), New York NY-1 (U36803), Bayou (L36930), and Black Creek Canal (L39950). Similar phylogenetic trees were obtained using different methods, and the results presented are based on the neighbor-joining method using the Kimura 2-parameter algorithm. We also performed phylogenetic analyses on amino acid sequences, which had similar results to the nt sequences.

RESULTS

Distribution of Rodent Population

There were a total of 5,461 rodents of 16 species captured in the various defined geographic regions of Taiwan. They were identified by species and each rodent was tested for hantavirus infection. The majority of the rodents were obtained from the four international seaports (2,082, 38.1%) (Table I). In contrast, relatively few rodents were captured in rural and mountainous regions during the study period.

R. norvegicus was the most common species captured in all regions in Taiwan, even in rural areas. *R. rattus* and *S. murinus* were also very common, though in some regions these species were not found (Table I and Fig. 1). Notable exceptions were found on two isolated islands, (Kinmen and Orchid Islets), one domestic seaport (Nanliaou), and in two mountainous regions (Mt. Ali and Tenlang). *R. flavipectus* was the species indigenous to Kinmen and *R. mindanensis* was the species indigenous to the Orchid Islets, which accounted for more than 90% of all rodents captured on these two islands. Rodent distribution in mountainous regions was obviously different from that in low-land regions. In Tenlang, *A. semotus* was the dominant species, and, in Mt. Ali, *N. coninga* was the dominant species. Interestingly, the rodent distribution in Nanliaou, a domestic seaport, was totally different from that in other regions in Taiwan. Though it remains to be proven, this observation prompts us to suggest two possibilities. First, it might indicate a recent invasion of new rodent species from China resulting in a change of rodent distribution because Nanliaou has been known to be the major seaport for direct but illegal trade between fishermen of Taiwan and mainland China since 1990. Second, because this domestic seaport was built very recently, it is possible that the rodent distribution at Nanliaou has had only minor change and remains similar to that 10 years ago, when Nanliaou was a rural village. The species distribution and number of rodents captured at different months in the same regions were almost identical (data not shown), probably due to the lack of seasonal variation in temperature in low-land regions of Taiwan. It is interesting that almost no *A. agrarius* was captured in Taiwan (except at the CKS International Airport), despite sampling in two rural regions (Chusan and Yuzen) (Table I and Fig. 1). Multiple efforts have been made to confirm this observation, and similar results were found.

Seroprevalence of Hantavirus in the Rodent Population

R. norvegicus was the rodent species most frequently infected with hantavirus, with an antibody prevalence of more than 20% in international seaports (Table I). However, the infection rate in *R. norvegicus* varied markedly between domestic seaports in northern and southern Taiwan. The infection rate of hantavirus in northern domestic seaports (Suao and Padoze, 8.1%) was significantly lower than that (23%) in northern international seaport (Keelung) ($P < 0.01$). Furthermore, the trend of seropositivity from Keelung to Padoze and Suao (23%, 12.1% and 7.4%) decreased with an increasing distance between Keelung and Padoze or between Keelung and Suao ($P < 0.01$ for trend test) (Fig. 1). In contrast, hantavirus infection in *R. norvegicus* captured in southern domestic seaports (Pingtung and Tongkong) was more common than that in the southern international seaport (Kaoshong) ($P < 0.01$).

In contrast to the CKS International Airport in northern Taiwan, *R. norvegicus* in the Kaoshong International Airport in southern Taiwan, which is located in metropolitan Kaoshong City (including Kaoshong international seaport), showed a comparable prevalence of hantavirus infection as that in Kaoshong seaport (Table I). A very low seroprevalence of antibody was found in *R. norvegicus* in the two rural areas of Chusan and Yuzen (Table I), revealing little evidence of hanta-

TABLE I. The Distribution of Rodent Population and the Prevalence of Antibody to Hantavirus in Different Locations in Taiwan, 1994–1995

Location	*R. norvegicus*	*R. rattus*	*R. losea*	*B. indica*	*S. murinus*	*A. agrarius*	Other	Total
International seaport								
Keelung	23.0 (43/187)	13.0 (16/123)	—	0.0 (0/4)	0.9 (1/113)	—	—	14.1 (60/427)
Taichung	19.7 (72/366)	0.0 (0/5)	—	—	0.0 (0/68)	—	0.0 (0/9)	16.1 (72/448)
Kaoshong	23.7 (92/388)	9.3 (4/43)	—	40.0 (2/5)	1.7 (4/241)	—	0.0 (0/3)	15.0 (102/680)
Hualien	25.0 (41/164)	0.0 (0/57)	2.7 (4/148)	4.2 (1/24)	0.0 (0/113)	—	0.0 (0/21)	8.7 (46/527)
Domestic seaport								
Suao	7.4 (14/189)	2.1 (4/188)	—	—	0.0 (0/57)	—	0.0 (0/24)	3.9 (18/458)
Padoze	12.1 (4/33)	22.8 (13/57)	—	—	0.0 (0/15)	—	—	16.2 (17/105)
Nanliaou	0.0 (0/4)	0.0 (0/5)	0.0 (0/22)	5.9 (1/17)	0.0 (0/42)	0.0 (0/10)	0.0 (0/3)	1.0 (1/103)
Tongkong	44.1 (30/68)	0.0 (0/7)	—	—	3.8 (2/53)	—	—	25.0 (32/128)
International airport								
CKS	0.0 (0/25)	0.0 (1/18)	0.0 (0/92)	0.6 (1/176)	0.0 (0/43)	0.0 (0/55)	0.0 (0/18)	0.2 (1/427)
Kaoshong	26.0 (57/219)	12.2 (5/41)	—	—	2.0 (3/150)	—	—	15.9 (65/410)
Small city								
Pingtung	32.8 (24/73)	8.3 (1/12)	—	0.0 (0/11)	2.3 (3/128)	—	—	12.5 (28/224)
Rural region								
Chusan	6.7 (1/15)	0.0 (0/1)	0.0 (0/3)	0.0 (0/1)	0.0 (0/79)	0.0 (0/2)	0.0 (0/3)	1.0 (1/104)
Yuzen	0.0 (0/23)	—	0.0 (0/3)	0.0 (0/20)	0.0 (0/49)	—	0.0 (0/4)	0.0 (0/99)
Mountainous region								
Tenlang	—	—	—	—	—	—	0.0 (0/58)	0.0 (0/58)
Mt. Ali	—	0.0 (0/1)	0.0 (0/3)	—	—	—	0.0 (0/61)	0.0 (0/65)
Isolated island								
Kinmen	22.0 (9/41)	0.0 (0/4)	—	—	0.0 (0/53)	—	0.9 (5/563)*	2.1 (14/661)
Matzu	28.6 (16/56)	0.0 (0/3)	—	—	0.0 (0/79)	—	—	11.6 (16/138)
Orchid Islets	—	—	—	—	—	—	0.0 (0/202)†	0.0 (0/202)
Penghu	45.5 (5/11)	0.0 (0/1)	—	—	0.0 (0/75)	—	0.0 (0/5)	5.4 (5/92)
Shaolouchous	33.3 (6/18)	15.8 (3/19)	0.0 (0/1)	—	0.0 (0/65)	—	50.0 (1/2)	9.5 (10/105)

**R. flavipectus* was the dominant rodent species captured in Kinmen.
†*R. mindanensis* was the dominant rodent species captured in Orchid Islets.

virus infection. Hantavirus infection was very common in *R. norvegicus* on the isolated islands of Kinmen and Matzu (Table I), similar to that of the international seaports. This is consistent with the fact that transportation between these islands (except the Orchid Islets) and the international seaports (except Haulien) is very frequent.

Seroprevalence of hantavirus showed an increasing trend from 1994 to 1995 (7.2% in 1994 and 10.2% in 1995 in all rodents captured). The most striking increase was found in the eastern international seaport, Hualien. The seroprevalence in *R. norvegicus* in Hualien was only 1.5% (1/74) in 1994 but greatly increased to 44% (40/90) in 1995, suggesting a recent invasion of hantavirus.

Evidence of hantavirus vector specificity was demonstrated by our finding that an extremely low hantavirus seroprevalence in *S. murinus,* the second most common rodent species captured in the present study, while seroprevalence in *R. norvegicus* found at the same geographic region was high.

Active Hantavirus Infection in Rodents

RT-PCR testing of individual rodents that were serologically positive for hantavirus antigen showed a high proportion with active infection. About 40% of the *R. norvegicus* and *R. rattus* found to be antibody-positive were subsequently found to have amplifiable hantavirus sequences in their lung tissues (33.9% and 44.5%, respectively). Furthermore, these positive results were all from the RT-PCR using primers specific for HTN/SEO strains, while none of the RT-PCR using primers for PUU/PH revealed positive results. No significant differences in the proportion with active infection were found between rodents from different regions or from rodents captured at different times (data not shown).

Particular attention was focused on specific rodents that were not the natural reservoir for hantavirus. Thus, it is interesting to find that 30% of seropositive *R. flavipectus* captured in Kinmen had positive RT-PCR findings for HTN/SEO.

Sequence Comparison and Phylogenetic Analyses

To understand the hantavirus strains prevailing in Taiwan, and, furthermore, to define the source of infection, we randomly selected viruses from rodents showing active infection (positive RT-PCR), and these virus sequences were further subjected to phylogenetic analyses. To ensure virus strains from different geographic regions were included in the analyses, we purposely selected a few strains from each region. On the basis of the tree of Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) (produced by MEGA version 1.01) (data not shown), we excluded similar sequences from the same regions, and only included 14 representative sequences (Fig. 2). To reconstruct the phylogenetic tree, we also obtained 13 representative sequences of hantavirus belonging to different serotypes from the Genetics Computer Group (GCG), and these sequences served as a basis for comparison in the following analyses.

To obtain preliminary insight about hantavirus in Taiwan, a sequence comparison of nt differences was made on a 201-nt fragment of the G2 glycoprotein-encoding region of the virus M2 genome segment. The sequence diverged 2%–25% at the nt level among the 14 Taiwanese hantaviruses, and 11 of these sequences were similar (<10% difference). Furthermore, consistent with the distribution of the rodent population in Taiwan showing *R. norvegicus* as the dominant species, 11 sequences were found to be consistent with the SEO strain. However, unexpectedly, the sequence homology among the SEO strain in Taiwan was found to be 90–95%, which was lower than the 95–99% sequence homology found in different prototypes of SEO strains in other parts of the World [Schmaljohn, 1996; Xiao et al., 1994]. The other three sequences revealed a notable genetic diversity (Fig. 2), differing from any of the 11 Taiwanese hantaviruses by at least 15% (mostly >20%). Interestingly, two of them were from rodents captured on Kinmen island.

The phylogenetic relationship inferred by the neighbor-joining method based on 500 bootstrap replications is shown in Figure 3. The validity of this tree was confirmed by a >50% bootstrap confidence limit in each branch node and by correct grouping of known hantavirus strains of different serotypes. Phylogenetic analyses of the sequence differences indicated that all hantaviruses in Taiwan, except one (C41015), was most closely related to the prototype SEO strains (SR-11 and R22), and no HTN strain was recovered from any rodent species indigenous to Taiwan island. The only exception (C41015) was from Kaoshong international seaport, in which the sequence was close to a Thai strain.

Of particular interest is the hantavirus in the isolated islands studied. The virus sequences from rodents captured on Matzu and Penghu were similar to the SEO strain found in the international seaports in Taiwan. However, an interesting diversity of hantavirus was found in Kinmen, where three distant sequences were identified. Two of them were classified in the cluster of SEO strain (Fig. 3), but a significant difference (>20% nt difference) was observed in one (KM372) of these two. The remaining one (KM431) represented a distinct lineage, and was closely related to the HTN strain, which was the dominant strain recovered from the rodent species (*A. agrarius*) indigenous to southeastern China. However, only a three-base (1.5%) difference was observed between KM431 and the HTN prototype (76-118), but the 76-118 strain was originally recovered from a rodent species in Northern Asia (Korea) [Lee et al., 1978; Schmaljohn et al., 1983], not southeastern China. Given that hantaviruses do not adapt readily to new host hosts [Schmaljohn and Hjelle, 1997], it is interesting to find that KM431 was recovered from the rodent species indigenous to Kin-

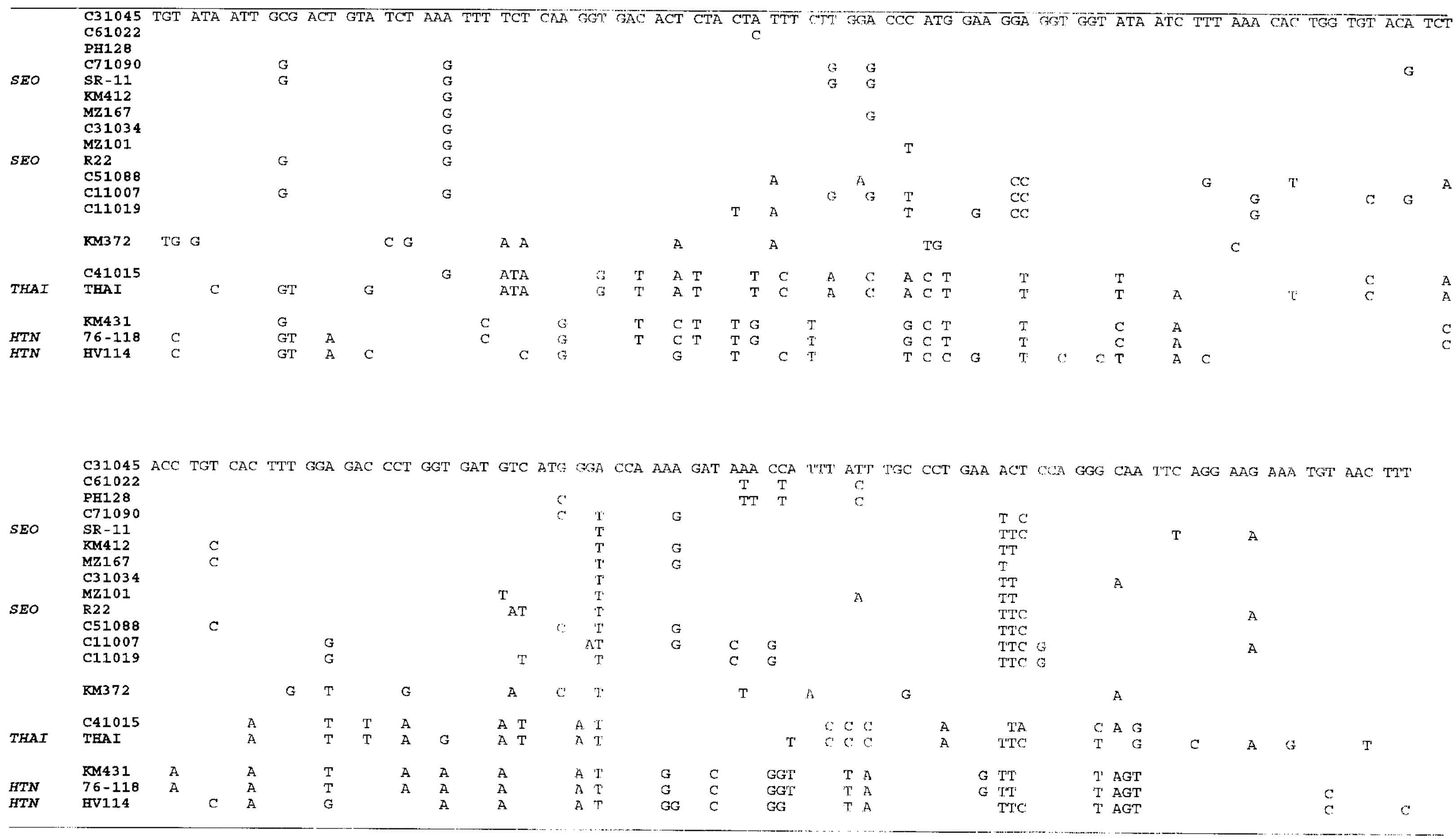

Fig. 2. Comparison of nucleotide sequences of Taiwanese hantaviruses and previously characterized hantaviruses. The sequences of the virus were within 201 bp of the RT-PCR amplified fragment (M segment encoding G2 glycoprotein of hantavirus). Sequence differences are shown relative to a typical SEO strain in Taiwan. All sequences are in the viral complementary DNA (+) sense.

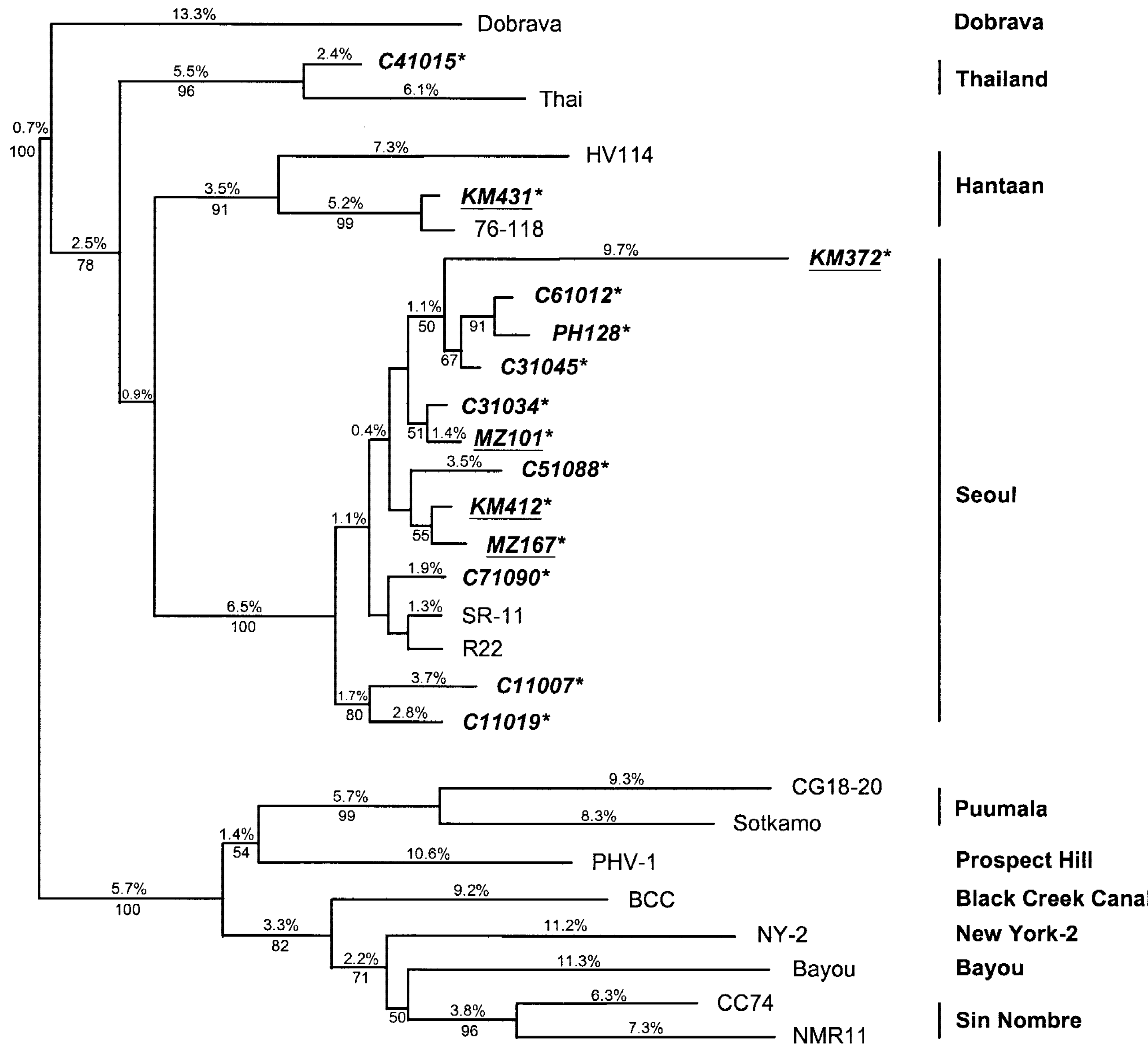

Fig. 3. Phylogenetic relation of the hantavirus isolated from rodent population trapped in Taiwan to previously characterized hantaviruses. Phylogenetic analysis of the virus sequence differences within 201 bp of the RT-PCR amplified fragment (M segment encoding G2 glycoprotein of hantavirus) was performed by the neighbor-joining method. The horizontal distances represent the number of nucleotide step differences (indicated upper to the line) present between branch nodes and virus. Bootstrap confidence limits exceeding 50 are indicated lower to each branch. (*), Taiwanese strains; underline: hantavirus from Kinmen and Matzu.

men (*F. flavipectus*), which is not the known natural reservoir for the HTN hantavirus strain.

DISCUSSION

With an annual incidence of 50,000–100,000 cases of HFRS per year caused by hantavirus in Mainland China [Schmaljohn and Hjelle, 1997; Lee, 1996], we are particularly concerned about the prevalence of infection, the mode of transmission, and the incidence of hantavirus HFRS in Taiwan. Rodent distribution, human behavior and viral pathogenicity all represent important determinants involved in this issue. The present study was conducted to examine the hypotheses that the hantavirus strain prevailing in Taiwan mainly belongs to the mild strain, the Seoul (SEO) strain, and the absence of severe disease was related to the absence of HTN. The combined results of our molecular and epidemiologic observations support our hypotheses.

The epidemiology of hantavirus infection in Taiwan is different from that in China. Only the SEO strain was identified in Taiwan, but, in China, both the HTN and SEO strains concurrently prevail [Chen et al., 1986]. Infection rates (seroprevalence) of the SEO strain were very different between *R. norvegicus* in China and Taiwan. In sharp contrast to <5% in China

[Chen et al., 1986; Chinese Academy of Preventive Medicine, 1992], more than 20% of *R. norvegicus* were found to have antibody against hantavirus. Though the possibility that different behavior of virus-carrying rodents (*R. norvegicus*) resulting in different infections rates can be a possible explanation, it is also likely that this is a reflection of the presence of differences in viral characteristics in association with infectivity between SEO strains from Taiwan and China. This difference, subsequently, might contribute to distinct seropositivity observed. Furthermore, the seroprevalence of hantavirus (SEO) infection in *R. norvegicus* in Kinmen and Matzu closely resembles that in the international seaports in Taiwan. More importantly, the SEO identified in Kinmen and Matzu was genetically linked to Taiwanese SEO strains, suggesting that frequent cross-strait transportation via boats between these islands and Taiwan represents an important transmission mode for both rodents and viruses to these isolated islands. Taken together, we suggest that Taiwanese hantavirus might not have been transmitted from China, and that geographic barrier between Taiwan (including Kinmen and Matzu) and China could explain these differences in epidemiologic and virologic profiles of hantavirus infection. A similar situation exists in Korea on Jeju and Chin islands. Both islands are close to the cost of Korean Peninsula where HFRS is endemic, but both are non-endemic regions of HFRS, and only seronegative *A. agrarius* have been found [Lee, 1989]. However, the unique HTN-like SEO strain (KM372) found in Kinmen showed distinct sequences compared to other Taiwanese strains. This strain might be a hantavirus directly transmitted from the southeastern provinces of China via illegal trade.

These data clearly implicate that the prevalence of *R. norvegicus,* which is the major rodent reservoir for SEO, was the major determinant contributing to the strain of hantavirus and the severity of virus-associated HFRS in Taiwan. The finding that no HTN was identified might be mostly attributable to a small number of suitable rodent reservoirs, i.e., *A. agrarius,* in Taiwan. On the basis of the epidemiology of HTN, the predominance in male subjects associated with agricultural activities of HTN virus-associated HFRS is strongly influenced by *A. agrarius* preference to live in the particular rural environment of large-scale cultivation and growth of high-stalk crops including wheat and sorghum. This agricultural activity may present a higher risk of inhalation of aerosolized rodent excreta or infected particulates. However, since the economic bloom in the 1970s in Taiwan, this kind of rural environment and agricultural activity have been rare in Taiwan, even in rural regions such as Chusan and Yuzen in this study. Therefore, the possibility of exposure to *A. agrarius* and of being infected with HTN has been minor. Our inference can be supported by the distinctive rodent distribution at the two international airports. The CKS International Airport was built in the 1960s, when the CKS airport area was still an agricultural environment. Since then, the CKS area has been specified as a conservation zone due to air transportation safety concerns, and the ecoenvironment has remained only modestly changed if at all. Consequently, the rodent distribution in the CKS airport area was different from the current rural areas in Taiwan, and *A. agrarius* remained to be the dominant rodent species in this area. Even though, no HTN was found in *A. agrarius* in the CKS airport area. In contrast, the Kaoshong airport was built recently, and is located in the metropolitan area of Kaoshong City. Therefore, the rodent distribution at Kaoshong airport was similar to that of Kaoshong seaport.

Our finding that only SEO was prevalent in Taiwan and that no HTN could be identified was contradictory to a previous seroepidemiologic study by Kao et al. [1996], in which 6.2% (403/6536) of human serum specimens randomly sampled from Taiwanese residents showed detectable HTN antibodies. The seroassay used in Kao's study was a relatively non-specific method, indirect immunofluorescence, used only the prototype HTN virus (76-118)-infected Vero E6 cells as the viral antigen. Therefore, antigenic cross-reactivity found among HTN and SEO strains may explain this inconsistency. The problem of using a nonspecific assay might create a false-positive result. This possibility is indicated by the highest reported seropositivity rate (>20%) for humans and rats being observed on the Orchid Islets in Kao's study. However, in our study, we found no evidence of hantavirus infection in rodents captured on the Orchid Islets, based on detection of antibody against specific antigens. Moreover, we confirmed our finding by the observation that, on the Orchid Islets, no virus sequences were found in further RT-PCR in all seronegative rodents and that no antibody was detected in 300 residents on the Orchid Islets (our ongoing study, unpublished results).

The only HTN strain was found in Kinmen. However, it is interesting that the sequence of this unique HTN was very similar to that of the prototype of HTN (HTN strain 76-118) [Lee et al., 1978; Schmaljohn et al., 1983] originally isolated from rodents and HFRS patients in remote northern Asia (Korea). Perhaps equally interesting is that this HTN was not recovered from its predominant natural reservoir (*A. agrarius*), but was found in *R. flavipectus,* the only predominant rodent species in Kinmen. Because no HTN has ever been used for experimental purposes in Taiwan and all the experiments of this study were conducted in a local laboratory, the possibility of laboratory errors such as PCR contamination or misidentification of rodent species can be mostly excluded. Instead, this observation might be an example of "spillover" host infection, a secondary host infected through contact with the primary host [Schmaljohn and Hjelle, 1997].

The combined epidemiologic and molecular findings yield interesting clues into the transmission mode of hantavirus infection in Taiwan. A gradient of SEO seropositivity in the rodent species (*R. norvegicus*), being

much higher in international seaports than in rural regions and intermediate in some domestic seaports (Padoze and Suao) suggests that hantavirus invasion into Taiwan was mainly via international sea transportation. The geographic features characterizing northern Taiwan, where hills and mountains are located among the Keelung, Suao and Padoze seaports, are different from those of the domestic seaports in southern Taiwan (Pingtung and Tongkong), which are located in the neighborhood of the Kaoshong international seaport (Fig. 1). The proximity of these southern seaports and the lack of geographic features such as mountains provide an explanation for the finding that comparable rates of hantavirus infection were observed in *R. norvegicus* in these seaports. In addition, since the Kaoshong international seaport is the largest one in Taiwan, rodents there might have a higher risk of exposure to a variety of different strains of hantavirus, which may explain why the only non-SEO virus in Taiwan, a hantavirus of Thai strain, was found at the Kaoshong seaport.

An increase in seropositivity of hantavirus in rodents captured in different years (1994 and 1995) in our study suggests the possibility that a hantavirus's invasion into a group of susceptible rodents occurred only recently, because an equilibrium between virus adaption and their host has not been completely reached yet. We also examine the possibility of a recent invasion of hantavirus into Taiwan by showing a higher weighed average of synonymous (KS) vs. nonsynonymous (KA) substitution [Li, 1993] among Taiwanese SEO strains (calculated by the software kindly provided by Dr. WH Li at the University of Texas, Houston). For example, the KS and KA between two SEO strains in Taiwan (C61012 and C31034 in Fig. 3) are 0.089 and 0.041, respectively. In general, the KS represents random mutation, and the KA represents selection pressure. Taken together, these findings suggest that the time of hantavirus invasion into Taiwan might have occurred recently so that evolutionary pressure in local environment has been unable to impact the virus yet.

Our study yields interesting insights into the transmission of hantavirus in Taiwan. By viewing the history of HFRS reported in Japan or Hong Kong, which are geographically adjacent to HFRS-endemic areas (Korea and China), it appears to be intriguing that no HFRS has ever been diagnosed in Taiwan. The finding that hantavirus infection has occurred in Japan and Hong Kong [Lee, 1996] suggests that a geographic boundary preventing the spread of this rodent-born virus can be breached probably by direct contact via trade. It, therefore, becomes more interesting to observe the secular changes of hantavirus infection in Taiwan, and examine whether the HTN in China can be transmitted to Taiwan as a result of recent relief of tension between Taiwan and China since the 1990s.

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